PATENT
Docket No.: **201487/1070 (KUV-101PCT-US)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) :	Amagai et al.	)	Examiner: Q. Janice Li
Serial No. :	09/937,739 based on PCT/JP00/02023	)	
Cnfrm. No. :	5390	)	Art Unit: 1632
Filed :	March 30, 2000	)	
For :	AUTOIMMUNE DISEASE MODEL ANIMAL	)	

DECLARATION OF MASAYUKI AMAGAI UNDER 37 CFR § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, MASAYUKI AMAGAI, pursuant to 37 C.F.R. § 1.132, declare that:

1. I received a MD from Keio University School of Medicine, and a PhD from Keio University Graduate School of Medicine.

2. I was the Visiting Research Fellow at Dermatology Branch, National Cancer Institute, National Institutes of Health from 1989 to 1992, and the Instructor at Department of Dermatology, Ehime University School of Medicine from January to July, 1996.

3. I am currently the Assistant Professor at Department of Dermatology, Keio University School of Medicine.

4. I am a co-inventor of the above-identified patent application.

5. I present this declaration to demonstrate that researchers could prepare a mammalian model of an autoimmune disease by transferring naïve (i.e., non-activated) immune cells from a donor mammal deficient in a gene encoding an antigen protein of an autoimmune disease to a recipient mammal in accordance with the disclosure of my above-identified patent application and, in fact, have done so. In

particular, I am submitting this declaration to describe experiments which show that it is possible to make an autoimmune animal model for pemphigus vulgaris by transferring naïve immune cells from a $Dsg3^{-/-}$ donor mammal to a recipient mammal expressing the Dsg3 protein, as claimed in my above-identified application.

6. To determine whether the step of immunizing a $Dsg3^{-/-}$ mouse with recombinant Dsg3 (rDsg3) protein is critical for constructing a model mouse that produces anti-Dsg3 IgG and exhibits the phenotype of pemphigus vulgaris, I conducted the transfer of splenocytes from a naïve $Dsg3^{-/-}$ mouse (i.e., not immunized with Dsg3 protein) into a recipient mouse. $DSG3^{-/-}$ mice were prepared by mating male $DSG3^{-/-}$ mice with female $DSG3^{+/-}$ mice, and were used as donors (Koch et al., J. Cell Biol. 137:1091-1102 (1997)) (attached hereto as Exhibit A). $RAG2^{-/-}$ mice, which had been obtained by back-crossing with B6.SJL-$ptpr^c$ over 10 generations, were provided by Taconic (German Town, NY) (Schulz et al., J. Immunol. 157:4379-4389 (1996)) (attached hereto as Exhibit B), and were used as recipients. Such $RAG2^{-/-}$ mice express Dsg3 protein, but do not reject transplanted splenocytes, because they are deficient in rearrangement of T cell receptor genes and immunoglobulin genes, thereby having neither mature T cells nor B cells.

7. Splenocytes were isolated from naïve $Dsg3^{-/-}$ mice by a conventional method. To perform the adoptive transfer of splenocytes, monocytes were isolated from the spleens of $DSG3^{-/-}$ mice and re-suspended in complete RPMI 1640 medium (Nissui Pharmaceuticals, Tokyo) containing 10% fetal bovine serum, 0.21% sodium bicarbonate solution (w/v), 2 mM L-glutamine (GIBCO), and antibiotics. About 2×10^7 or about 5×10^7 splenocytes were suspended in PBS and transferred into a $RAG2^{-/-}$ mouse via a caudal vein by intravenous injection (each n=20). The production of antibody was tested by ELISA using rDsg3 as a coating antigen.

8. To carry out an ELISA assay for blood IgG against mouse Dsg3 protein (mDsg3), a 96-well microtiter plate was coated with 100 µl of 5 µg/ml purified mouse rDsg3 at 4°C overnight. Recipient serum samples were diluted 50 to 5,000 times and then incubated on the 96-well ELISA plate at room temperature for 1 hour. After the samples were incubated with peroxidase-conjugated anti-mouse IgG antibody (MBL, Nagoya, Japan) at room temperature for 1 hour, the coloring reaction was carried out by using 1 mM tetramethylbenzidine as a substrate for peroxidase

(Ishii et al., J Immunol 159:2010-2017 (1997) (attached hereto as Exhibit C); Amagai et al., Br J Dermatol 140:351-357 (1999) (attached hereto as Exhibit D)). The time course of OD_{450} change in each sample was analyzed in duplicate. Results in mouse #799, #800, and #317 which were transplanted with 2×10^7 splenocytes per recipient mouse, and #310 and #337 which were transplanted with 5×10^7 splenocytes per recipient mouse are shown in Figure 1 (attached hereto as Exhibit E). Except for #317, anti-Dsg3 IgG was detected in the blood of recipient $RAG2^{-/-}$ mice at day 14 after the transfer of $DSG3^{-/-}$ splenocytes. The antibody production was rapidly increased and reached a plateau around day 28. Antibody production then continued permanently. The sustained antibody production was observed for 6 months or more as long as the mice were alive.

9. Further, the production of antibody against Dsg3 protein was tested by immuno-fluorescent staining of cultured keratinocytes. Mouse keratinocytes from cell line PAM212 (Yuspa et al., Cancer Res. 40:4694-4703 (1980)) (attached hereto as Exhibit F) were incubated with recipient mouse serum sample diluted 20-fold with DMEM containing 10% fetal calf serum at 37°C under humid air containing 5% CO_2 for 30 minutes. Subsequently, the cells were washed with PBS(-), fixed with 100% methanol at -20□ for 20 minutes, and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (DAKO, Copenhagen, Denmark) at room temperature for 30 minutes. The stain was observed using a fluorescence microscope (Nikon, Eclipse E800). As a result, *in vivo* IgG deposition was found on the cell surface of stratified squamous epithelium keratinocytes in the recipient $RAG2^{-/-}$ mouse. This result is shown in Figure 2 (attached hereto as Exhibit G).

10. In recipient mice that were transplanted with 2×10^7 splenocytes per mouse, the production of anti-Dsg3 antibody was detected at 2 weeks after the transfer in 12 out of the 20 mice examined by ELISA, and the phenotype of pemphigus vulgaris was apparent at 4 weeks after the transfer in 11 mice. In recipient mice that were transplanted with 5×10^7 splenocytes per mouse, the titer of the antibody was increased at 2 weeks after the transfer in 16 out of the 20 mice examined, and the phenotype was apparent at 3 weeks after the transfer in 15 mice. In the mice exhibiting the pemphigus vulgaris phenotype, IgG deposition was found in the inter-cell region of the mucous membrane of the hard palate, as shown in Figure 3A

(attached hereto as Exhibit H). Acantholysis immediately above the basal layer, which is a typical characteristic of pemphigus vulgaris, was also observed, as seen in Figure 3B (attached hereto as Exhibit H). The mice showing the phenotype described above also exhibited extensive hair loss, submaxillar and plantar elosion, and scab-formation, which are characteristics of the major phenotype of pemphigus vulgaris. This is shown in Figure 4 (attached hereto as Exhibit I).

11. Accordingly, it is apparent that splenocytes of a naïve $Dsg3^{-/-}$ mammal are capable of being activated by contact with the endogenous Dsg3 of a recipient mammal after the transplantation of non-immunized splenocytes from the $Dsg3^{-/-}$ mammal to the recipient mammal. It is further apparent that the production of anti-Dsg3 antibody and exhibition of the phenotype of pemphigus vulgaris is induced in the recipient mammal.

12. As demonstrated by all the foregoing, it is possible to make an autoimmune non-human mammalian model by transplanting immune cells from a naïve antigen gene-deficient donor to a recipient, thereby inducing anti-antigen antibody production in the recipient animal, which results in the creation of an autoimmune disease condition phenotype in the recipient mammal, as claimed in my above-identified application.

13. I hereby declare that all statements made herein of my own knowledge are true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date : July 10, 2003 Masayuki Amagai

Masayuki Amagai